REMARKS

The present invention provides methods for identifying active proteins in a complex protein mixture (*e.g.*, a proteomic mixture). Complex protein mixtures are reacted with a single activity based probe (ABP—preferably an ABP that binds to active target proteins at a single site), and the resulting protein conjugates are proteolytically digested, providing probe-labeled peptides. Prior to the present invention (as reflected by the prior art cited by the Examiner, i.e., Aebersold and Cravatt), the skilled artisan would have expected that proteolytic digestion would result in a more complex protein mixture. In contrast, the present invention demonstrates that such proteolysis simplifies the complex protein mixture during subsequent analysis.

In preferred embodiments, ABPs are selected such that each active target protein forms a conjugate with a single ABP, preferably at a single discrete location in the target protein; thus, each conjugate gives rise to a single peptide labeled with a single probe. Enrichment separation or identification of one or more ABP-labeled peptides may be achieved using liquid chromatography and/or electrophoresis. Mass spectrometry may be employed to identify one or more ABP-labeled peptides by molecular weight and/or amino acid sequence. In particularly preferred embodiments, sequence information derived from one or more of the ABP-labeled peptide(s) may be used to identify the protein from which the peptide was originally derived. Variations of these aspects can involve comparison of two or more proteomes, *e.g.*, with a single ABP, or, when analysis comprises mass spectrometry, probes having different isotopic compositions.

Thus, methods of the present invention provide enhanced simplicity and accuracy in the identification of the active protein composition of a complex protein mixture.

No amendments are presented herein. Accordingly, claims 21-32 and 48-74 remain pending, with claims 21-32, 48-59 and 74 under active prosecution. The present status of all claims in the application is indicated in the Listing of Claims, which begins on page 2.

The restriction of claims 21-32 and 48-74 is respectfully traversed. It is respectfully submitted that no savings of PTO resources will be realized by maintaining the restriction requirement as presently asserted. Indeed, a thorough search of the Group II claims would, of necessity, involve a search of the subject matter of the Group I claims. Accordingly, reconsideration and withdrawal of the requirement for restriction are respectfully requested. In the alternative, rejoinder of the non-elected claims, upon final disposition of the elected claims, is respectfully requested.

However, in order to be fully responsive, Applicant hereby affirms the election of Group I (claims 21-32, 48-59 and 74). Non-elected Group II claims (i.e., claims 60-73) are retained in the application pending final resolution of the elected claims, and possible rejoinder at that time.

The withdrawal of the rejection of claims 21, 22, 24, 25, 27, 28 and 48 under 35 U.S.C. § 102(e) as allegedly being anticipated by Cravatt et al., U.S. 2002/0045194, is acknowledged with appreciation.

The rejection of claims 21-28, 30-32, 48-54, 56-59 and 74 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Aebersold et al., U.S. 2002/0076739, in view of Cravatt, is respectfully traversed. Applicant's invention distinguishes over the combination of Aebersold in view of Cravatt in at least the following ways:

(1) the combination of Aebersold and Cravatt does not teach the use of activity based probes wherein digestion of the protein sample is done prior to separation;
(2) the prior art contemplates the use of sets of isotopically labeled probes, rather than the use of a single activity based probe, as required by the present invention; and
(3) prior to the present invention, the belief in the art was that a single peptide would not provide accurate identification of proteins.

Specifically, Applicant's invention, as defined, for example, by claim 21, distinguishes over the combination of Aebersold in view of Cravatt by requiring a method for determining the

presence, amount, or activity of one or more active target proteins in a complex protein mixture, the method consisting essentially of:

(a) contacting said complex protein mixture with a single activity based probe that specifically binds predominantly to a single target site on one or more active target proteins;

(b) optionally binding said target protein(s) to a solid support;

(c) proteolyzing said active target protein(s) to produce a product mixture;

(d) separating said product mixture into two or more components, one or more of which consist essentially of peptides bound to said probe; and

(e) generating a signal from said peptides bound to said probe, wherein said signal is correlated to the presence, amount, or activity of said one or more active target proteins in said complex protein mixture.

Neither Aebersold nor Cravatt teach such a method for determining the presence, amount or activity of one or more active target proteins employing an activity based probe.

In the Amendment dated April 26, 2005, the claims were amended to define the invention with greater particularity and arguments were presented to distinguish the present invention over the Aebersold and Cravatt references. In the most recent Office Action (dated June 28, 2005), the Examiner asserts that the combination of Cravatt and Aebersold teach all of the elements of the invention, as claimed, curing the defects of the application of the Aebersold reference by itself because Cravatt "teaches the analysis of a single sample." However, the Examiner's assertion is not accurate. Cravatt does not contemplate a method for analyzing proteins in a single complex protein mixture, e.g. a proteome, wherein each element of the present invention, as required for example in claim 21, is described. Instead, Cravatt teaches that when a single activity based probe is used to label a protein sample, protein digestion is done after separation. See Cravatt paragraphs [0107] and [0183-0184]. Cravatt only contemplates protein digestion prior to separation when two or more protein samples are quantitatively compared using sets of

isotopically labeled activity based probes. See Cravatt paragraph [0128]. The present invention, wherein a single activity based probe is used to analyze a single protein sample, and protein digestion is done before separation, (as claimed, for example, in claim 21), is clearly distinct from the Cravatt method.

No combination of Aebersold and Cravatt suggests or discloses every element of the inventive methods as presently claimed. Cravatt is the result of combining the Aebersold method and the use of activity based probes (see Cravatt paragraph [0128]), wherein two samples are compared using two isotopically distinct activity based probes, and the resulting isotopically labeled peptides are analyzed using mass spectrometry. While the Cravatt method employs protein digestion prior to separation, the remaining method steps, as disclosed by Cravatt, are clearly different from the steps as required by the present claims (e.g., the use of a single activity based probe). Specifically, Cravatt discloses the use of isotopically labeled sets of probes. In contrast, the present invention employs single activity based probes. Furthermore, the Cravatt method is clearly directed to quantitative comparison of at least two protein samples, rather than the identification of a single protein sample with a single activity based probe, as required by the present claims.

Moreover, Applicant respectfully disagrees with the Examiner's assertion that:

> It would have been obvious to one of ordinary skill in the art to substitute the activity based probe such as taught by Cravatt et al for the probe of Aebersold et al because Cravatt et al teaches the use of a single probe in single and combined samples and also because Cravatt et al recognized the need for methods of measuring protein activity in proteomics. . . Therefore, a skilled artisan can have a reasonable expectation of success in incorporating an activity based probe taught by Cravatt et al in the method of Aebersold et al.

(See page 5, lines 5-15 of the Office Action; emphasis added.) Contrary to the Examiner's assertion, recognition of a need in the art is not the same as an expectation of success. Mere

recognition of a need may provide the motivation to try, but it clearly does not, without more, rise to the level of an expectation of success. The Examiner has provided absolutely no support from either Aebersold or Cravatt for the assertion that a skilled artisan would have an expectation of success in the combination of the Cravatt and Aebersold methods. Furthermore, the claims as presently presented are clearly not a combination of Aebersold and Cravatt (i.e., activity based probes), because the Aebersold method requires the use of a different probe (i.e., sets of isotopically distinct probes) for a different purpose (i.e., quantitative comparison of multiple samples).

In the section of the present Office Action labeled "Response to Arguments," Applicant respectfully disagrees with the Examiner's assertion that "applicant is arguing the references individually." (See page 9, lines 2-3 of the Office Action). Before one can consider the potential impact of a combination of references, one must look at the teaching of each reference individually—then, and only then, can one evaluate whether the asserted combination of references is logical, and if so, what a logical combination of the teachings would actually suggest to one of skill in the art.

Applicant further disagrees with the Examiner's assertion that "since the combination of Aebersold and Cravatt teach a single activity based probe the methods would produce fewer peptides." See page 9, lines 17-18 of the Office Action. It is clearly only with improper hindsight benefit of Applicant's disclosure that the above-quoted assertion can be advanced.

As discussed previously, labeling with the activity based probes of the present invention's methods produce fewer peptides than the use of the Aebersold probes. Contrary to the Examiner's assertion, the reason fewer peptides are produced by the present method is not because the present invention employs a single activity based probe and Aebersold employs multiple probes. Instead, fewer peptides are produced because activity based probes, as defined in the present invention, react with a specific amino acid side chain only when it is within a particular structural/functional context, i.e., an enzyme active site. For example, while a serine

hydrolase enzyme may have 60 serine residues in its amino acid sequence, the activity based probe of the present invention will only react with and label the single serine residue which is part of the "catalytic triad" at the enzyme active site. In contrast, the Aebersold probes, when similarly applied, will label each individual serine residue present.

The Examiner's further assertion that the features upon which Applicant allegedly relies (i.e., lower resolution, higher throughput separation methods such as LC or CE), are not recited in the present claims, is not relevant. These separation methods are available for use in the present inventive methods because, as a result of the use of activity based probes according to the present invention, fewer labeled peptides are produced. These inherent benefits result from the decreased peptide sample and do not need to be explicitly recited in the claims.

As discussed in detail in Applicant's previous response (Amendment dated April 26, 2005), Aebersold does not disclose or suggest a method for determining the presence, amount, or activity of one or more active target proteins in a complex protein mixture. As acknowledged by the Examiner, "Aebersold et. al. differ from the instant invention in failing to teach the probe is an activity based probe and the use of a single activity based probe." (See page 4, lines 11-12 of the Office Action). Thus, the Aebersold method (i.e., using sets of isotopically labeled probes) is distinct from the claimed invention in at least two significant ways: Aebersold discloses the use of a set (as opposed to a single probe) of isotopically labeled probes (as opposed to an activity based probe) for the quantitative comparison of protein samples.

The differences between the use of single activity based probes and sets of isotopically labeled probes are very significant. The activity based probes of the present inventive method label a single target site on each protein, resulting in a single labeled peptide from each protein after digestion. In contrast, the Aebersold probes label multiple sites on each protein, resulting in an increased number of peptides. Prior to the present invention, the standard belief in the mass spectrometry community was that a single peptide did not provide data with sufficient confidence to unambiguously identify a protein through automated sequence searching algorithms.

As discussed in substantial detail in Applicant's prior communications, the prior art has shown that when only a small number of peptides matched a particular protein (1-3 peptides), researchers manually inspected the MS data to determine test validity.[1] Because a typical mass spectrometry run generates on the order of 4000 spectra, manual analysis of each individual spectrum (at a rate of about 15 minutes per spectrum) would not be feasible if all data were expected to fall into the category of single site labeling of each protein.

In contrast to Aebersold, the use of activity based probes according to the present invention results in the labeling of a single site on each target; and subsequent proteolytic digestion does not increase the complexity of the sample. The probes employed by Aebersold are clearly distinct from the activity based probes employed in the practice of the present invention—because Aebersold employs sets of isotopic variants of the probes that label multiple sites on each target protein.[2] Each of the probes employed in the Aebersold method labels multiple sites on the target proteins because the Aebersold probes are generally reactive with a particular amino acid side chain (e.g., cysteine), and lack specificity for the context of the residue within the structure/function of the protein.[2] In contrast, the activity based probes of the present invention are reactive with a specific amino acid side chain only when it is within a particular structural/functional context (i.e., an enzyme active site). Thus, because the Aebersold method employs probes which do not label proteins with specificity, when a labeled sample according to Aebersold is digested, the number of labeled peptide species is substantially increased, typically at least 10X or greater, relative to the number of labeled proteins. In contrast, because the

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[1] See, for example, Florens et. al., *Nature* 419, 520-26 (2002) (especially the final paragraph of the methods section, which discusses the analysis of "low coverage loci" by visual inspection), Adkins et. al., *Molecular and Cellular Proteomics*, December, 947-55 (2002)(specifically the legend of Table I legend (p. 949), which expressly states that "When three or fewer peptides for an individual protein passed the criteria shown in Table I, the mass spectra for those peptides were inspected manually."), and Washburn et. al., *Nature Biotechnology*, 19, 242-47 (2001) (specifically the final paragraph of the experimental section, which states that "We manually confirmed each SEQUEST result from every protein identified by four or fewer peptides").

[2] Typically 5-30 sites—if, for example, the Aebersold "protein reactive group" is a cysteine, since the average protein in the complete human database has about 360 amino acids and cysteine has a relative abundance of 2.8%, the average number of cysteine residues per protein is 10.

methods of the present invention produce substantially fewer peptides, this allows for the use of separation methods not applicable or possible with the Aebersold methods, e.g., lower resolution, higher throughput separation methods such as CE or LC (instead of LCMS/MS, which is typically employed in the Aebersold methods).

Furthermore, as also discussed in substantial detail in Applicant's prior communications, activity based probes contemplated for use according to the present invention are typically larger than the Aebersold probes; the labeling of the active site of the protein with the probes result in very large peptides, allowing for mass spectrometry data collection only on higher charge states of peptide ions (+3, +4, +5). (The preceding passage is highlighted to call attention to the context in which the size of activity based probes employed in the practice of the present invention (relative to the Aebersold probes) is discussed—in contrast to the "out-of-context" discussion thereof at page 8, lines 13-19 of the Office Action). Previously, the belief was that only +1, +2, and in some cases +3 ions, provided data with sufficient quality to give a confident peptide sequence. Indeed, as shown in the prior art references provided with Applicant's prior communications, most laboratories only attempted to identify peptide sequences from +1 and +2 peptide ion data. The belief of those skilled in the art was that data from ions with higher charge states (e.g. +3, +4 and +5) could not accurately be used for protein sequence determination.[3]

Still further, the Examiner's assertion that features relied upon by Applicant (i.e., that ABP-labeled sample can be analyzed *in silico* (computationally) or that generally >95% of

[3] See, for example, Aebersold et al., *Chemical Reviews*, **101**, 269-95 (2001) (first paragraph on p. 278 states that "$[M+2H]^{2+}$ ions of peptides will produce tandem mass spectra of higher quality than those from either $[M+H]^{+}$ or $[M+3H]^{3+}$ peptide ions. The $[M+2H]^{2+}$ peptide ions fragmented under low-energy CID produce spectra…that are more readily interpreted than tandem mass spectra of $[M+3H]^{3+}$ and higher charge states"); Washburn et. al., *Nature Biotechnology*, **19**, 242-47 (2001), (the final paragraph of the experimental section states "Peptides identified by SEQUEST may have three different charge states (+1, +2, +3), each of which results in a unique spectrum for the same peptide."); Shen et. al., *Analytical Chemistry*, **76**, p 1134-44 (2004) (see the experimental section which provides the score criteria used for +1, +2, and +3 ions. Charge states higher than +3 were not searched); Florens et. al., *Nature*, **419**, 520-26 (2002) (see the experimental section thereof which provides the score criteria used for +1, +2, and +3 ions. Charge states higher than +3 were not searched); and Adkins et. al., *Molecular and Cellular Proteomics*, 947-55 (2002) (see the experimental section thereof which provides the score criteria used for +1, +2, and +3 ions. Charge states higher than +3 were not searched.)

peptides derived from tryptic digest of ABP labeled proteins are non-redundant) are not included in the claims is irrelevant. Such inherent benefits in the claimed method are not required to be explicitly recited in the claim.

In view of the above background discussion providing context for the work described by Aebersold, it is respectfully submitted that further reliance on Cravatt does not cure the deficiencies of Aebersold. The combination of Aebersold and Cravatt does not teach the invention as required by the pending claims, namely the quantitative analysis of multiple proteins in a sample with a single activity based probe wherein protein digestion precedes separation.

Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. § 103(a) as allegedly being unpatentable over Aebersold et al. in view of Cravatt are respectfully requested.

The rejection of claims 29 and 55 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Aebersold et al. and Cravatt, and further in view of Little et al., U.S. 2003/0003465, is respectfully traversed. Applicant's invention, as defined, for example, by claim 29, distinguishes over the combination of Aebersold and Cravatt in view of Little, by requiring a method for determining the presence, amount, or activity of one or more active target proteins in a complex protein mixture, the method consisting essentially of:

(a) contacting the complex protein mixture with a single activity based probe that specifically binds predominantly to a single target site on one or more active target proteins;

(b) optionally binding said target protein(s) to a solid support;

(c) proteolyzing the active target protein(s) to produce a product mixture, wherein prior to proteolyzing, the one or more active target proteins bound to the probe are bound to a solid support;

(d) separating the product mixture into two or more components, one or more of which consist essentially of peptides bound to the probe; and

(e) generating a signal from the peptides bound to the probe, wherein the signal is correlated to the presence, amount, or activity of the one or more active target proteins in the complex protein mixture.

As discussed above, neither Aebersold nor Cravatt, taken alone or in combination, are capable of rendering the present invention, as defined in the current claims, obvious. Indeed, as acknowledged by the Examiner, "Aebersold et al and Cravatt et al differ from the instant invention in failing to teach prior to the proteolyzing step, the [sp—that] one or more active target protein[(s)] bound to the probe are bound to a solid support." (See page 6, lines 6-8 of the Office Action).

Further reliance on Little is unable to cure the deficiencies of Aebersold or Cravatt, taken alone or in combination, as Little addresses none of the acknowledged limitations of Aebersold or Cravatt, taken alone or in combination, as discussed in detail above.

Accordingly, as described herein, the present invention distinguishes over the prior art in numerous ways, including, for example,

(1) the combination of Aebersold and Cravatt does not teach the use of activity based probes wherein digestion of the protein sample is done prior to separation;
(2) the present invention employs a single activity based probe, rather than sets of isotopically labeled probes; and
(3) prior to the present invention, the belief was that a single peptide would not provide accurate identification of proteins.

Therefore, in view of the remarks presented herein and the remarks and amendments of record, reconsideration and withdrawal of the rejection under 35 U.S.C. § 103(a) as allegedly being unpatentable over Aebersold et al. and Cravatt and further in view of Little are respectfully requested.

In view of the above remarks, reconsideration and favorable action on all claims are respectfully requested. In the event any issues remain to be resolved in view of this communication, the Examiner is invited to contact the undersigned by telephone so that a prompt disposition of this application can be achieved.

Respectfully submitted,

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